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Daniel R. Perez *Editor*

Reverse Genetics of RNA Viruses

Methods and Protocols

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Preface

The International Committee on Taxonomy of Viruses (ICTV) classifies RNA viruses as those that belong to Group III, Group IV, or Group V of the Baltimore classification system and contain ribonucleic acid (RNA) as genetic material throughout their entire life cycle. Group III includes double-stranded RNA viruses (dsRNAs), whereas Groups IV and V contain single-stranded RNA viruses (ssRNAs) of positive and negative polarity, respectively. Positive sense RNA viruses (+ssRNAs) are those in which the RNA itself is translated by the host cell translation machinery and initiates an infectious cycle de novo. In contrast, negative sense RNA viruses (−ssRNAs) cannot be translated directly and require copying of the negative sense RNA into a positive sense RNA strand before the infection can proceed.

In biology, the term "forward genetics" is used to define an approach that seeks to find the genetic basis of a phenotype or trait. Forward genetics of RNA viruses implies imposing them to various stress conditions and then defining the genetic changes that occurred in the process. The term "reverse genetics" is an approach to unravel the function of a gene by establishing and analyzing the phenotypic effects of (artificially) engineered gene sequences. In case of RNA viruses, reverse genetics invariably requires the de novo reconstitution of the virus from a cDNA copy. Using molecular biology, cDNA copies of RNA viruses are cloned into a variety of vectors, most typically and in order of preference, plasmids, bacterial artificial chromosomes or bacmids, or recombinant viral vectors. The ability to further manipulate DNA elements encoding portions or entire cDNA copies of RNA viruses has revolutionized the manner in which these viruses can be studied and understood. Thanks to reverse genetics, it is possible to better define the molecular mechanisms that modulate pathogenesis, transmission, and host range of RNA viruses, to study virus evolution, receptor binding characteristics, virus entry, replication, assembly, and budding. Reverse genetics allows the development of novel vaccine strategies and to better test and/or develop alternative intervention strategies such as novel antivirals. Perhaps the initial perception is to think that reverse genetics of dsRNAs and +ssRNAs is easier than −ssRNAs; however, genome size, secondary RNA structures, genome segmentation, cryptic signal sequences, among other issues, make reverse genetics of all kinds of RNA viruses equally challenging.

This book *Reverse Genetics of RNA Viruses: Methods and Protocols* is a compilation of 16 chapters summarizing reverse genetics breakthroughs and detailed reverse genetics protocols. The book does not cover every reverse genetics protocol for every RNA virus. Instead, it does provide comprehensive protocols for those RNA viruses that were initially the most challenging to obtain and/or that were developed most recently. This book, of course, would not have been possible without the outstanding and most generous contributions of our authors who are leaders in their respective fields and that have shared their insights and step-by-step protocols to help you, our colleagues, with your own research endeavors. I hope you find this book helpful.

Athens, GA, USA ***Daniel R. Perez***

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Chapter 1

Reverse Genetics for Mammalian Orthoreovirus

Johnasha D. Stuart*, Matthew B. Phillips*, and Karl W. Boehme

Abstract

Reverse genetics allows introduction of specific alterations into a viral genome. Studies performed with mutant viruses generated using reverse genetics approaches have contributed immeasurably to our understanding of viral replication and pathogenesis, and also have led to development of novel vaccines and virus-based vectors. Here, we describe the reverse genetics system that allows for production and recovery of mammalian orthoreovirus, a double-stranded (ds) RNA virus, from plasmids that encode the viral genome.

Key words Plasmid-based reverse genetics, Reovirus, Double-stranded RNA virus, Recombinant virus, Viral reassortment, T7 RNA polymerase

1 Introduction

Viral mutants are powerful experimental tools. Analysis of mutant viruses has produced myriad breakthrough in our understanding of viral pathogenesis by illuminating how viruses replicate, alter host cell physiology, and modulate immune responses. Viral mutants can be derived using "forward genetics," where a selective pressure impairs one or more viral functions and requires the virus to adapt in order to replicate efficiently under the restrictive condition. Defining genetic changes that occur during adaptation can identify nucleotides in coding or noncoding regions of the viral genome that are associated with resistance to particular pressures. Forward genetics approaches are extremely effective for mapping the functions of viral proteins, but requires a selective pressure to restrict the virus and force genetic changes. In contrast, the ability to engineer viruses via reverse genetics enables the testing of properties for which a selective pressure is not available. Reverse genetics is the direct introduction of specific alterations,

*Johnasha D. Stuart and Matthew B. Phillips contributed equally to this work.

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including point mutations, insertions, and deletions, into a viral genome. In this chapter, we provide a protocol for generating mammalian orthoreovirus (reovirus) using a plasmid-based rescue system.

Reovirus is a member of the *Reoviridae* family of viruses that infect a range of host organisms, including mammals, birds, insects, and plants [1]. The *Reoviridae* family includes rotavirus, a common diarrheal pathogen among children [2]; bluetongue virus, an economically important agricultural pathogen that causes disease in sheep and cattle [3]; and mammalian orthoreovirus, a useful model for studies of dsRNA virus replication and pathogenesis [1]. Reoviruses were originally isolated in the 1950s [4]. Most people become infected by at least one of the three circulating reovirus serotypes during childhood [5]. Although reovirus infections are typically asymptomatic and self-resolve, they are implicated in a number of cases of central nervous system disease in children [1]. The three reovirus serotypes are represented by a prototype laboratory strain: type 1 Lang (T1L), type 2 Jones (T2J), and type 3 Dearing (T3D) [1]. Here, we provide a protocol for rescue of strains T1L and T3D using plasmid-based reverse genetics.

Reoviruses are non-enveloped, icosahedral viruses that contain a segmented genome consisting of ten ds RNAs [1]. The genomic dsRNA molecules are divided into three categories based on their molecular weight [6, 7]. The reovirus genome contains three large (L), three medium (M), and four small (S) genomic segments [8]. Each gene segment encodes a single viral protein except for the S1 segment, which encodes two proteins. The 5′ end of each reovirus positive-sense RNA contains a 7-methylguanosine cap, but the 3′ termini are not polyadenylated [9]. The negative-sense strand is complementary to the positive-sense strand and contains an unblocked phosphate at the 5′ end [10]. Two concentric protein shells, the outer capsid and core, comprise the virion particle [1]. Removal of outer capsid proteins during cell entry leads to deposition of a transcriptionally active core particle into the cytoplasm [11–13]. Nascent viral transcripts are extruded from channels at the icosahedral vertices of the core into the cytosol that are translated to make viral proteins [1]. Viral transcripts and newly synthesized viral proteins coalesce and create new cores in a neo-organelle called the viral factory. Viral transcripts are used as a template for synthesis of negative-sense RNAs within newly assembled core particles. Secondary rounds of transcription occur within the viral factories that amplify viral RNA and protein synthesis. Outer capsid proteins are added to the newly formed core particles to produce progeny virions that are released from cells by an unknown mechanism [1].

Transfection of cells with genomic dsRNA alone produces a minimal amount of viral progeny [14]. However, reovirus recovery is markedly increased by transfecting cells with viral ssRNA or

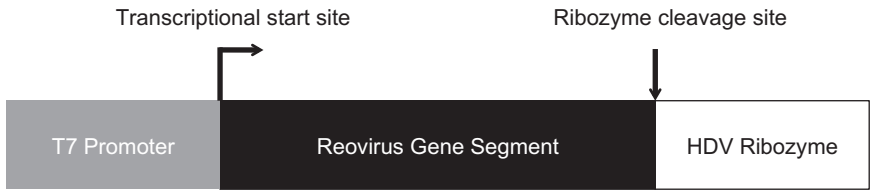

Fig. 1 Schematic of the reovirus T7 transcription cassette. Each reovirus gene segment cDNA is cloned into the plasmid vector downstream of a T7 polymerase promoter sequence and upstream of an HDV ribozyme sequence. The T7 transcriptional start site and HDV ribozyme cleavage site are indicated

dsRNA that was pre-incubated in rabbit reticulocyte lysate to allow translation of viral proteins, and then infecting with an attenuated helper reovirus [14]. Although infectious reovirus can be generated using the helper virus-based system, the technique is cumbersome and inefficient. Moreover, use of the helper virus increases the risk of reassortment between progeny virus and helper virus. However, the ability to rescue virus from ssRNA or melted dsRNA indicated that the positive-sense strand could be used to drive viral replication.

A plasmid-based reverse genetics system for reovirus was developed based on these observations [15]. Single plasmids encoding each of the ten reovirus gene segments were cloned downstream of bacteriophage T7 RNA polymerase promoter (Fig. 1). A hepatitis delta virus (HDV) ribozyme was inserted immediately downstream of the 3′ end. These features are designed to produce RNA transcripts that contain native reovirus 5′ and 3′ termini [16, 17]. The first-generation reovirus plasmid-based reverse genetics system relied on modified vaccinia virus strain DIs (rDIs) to supply T7 polymerase [15, 18]. To recover virus from plasmids, L929 cells were infected with rDIs prior to transfection with plasmids encoding all ten reovirus gene segments. Viable virus was recoverable within 48 h post-transfection [15]. Longer incubation times permitted amplification of rescued virus and yielded higher recovery titers. To increase rescue efficiency, a second-generation system employed baby hamster kidney cells that stably express T7 RNA polymerase (BHK-T7 cells) (Fig. 2) [19]. Use of BHK-T7 cells enhances the efficiency of reovirus recovery by ensuring that T7 RNA polymerase is expressed in every cell that receives plasmids. The second-generation system also uses plasmids that encode multiple reovirus gene segments to further enhance rescue efficiency by reducing the number of plasmids that must be taken up by a single cell. Currently, infectious reovirus can be recovered using as few as four plasmids [19].

Reovirus has long been at the forefront of viral genetics because the segmented genome enables mapping of serotype-specific phenotypic differences to an individual gene [1].

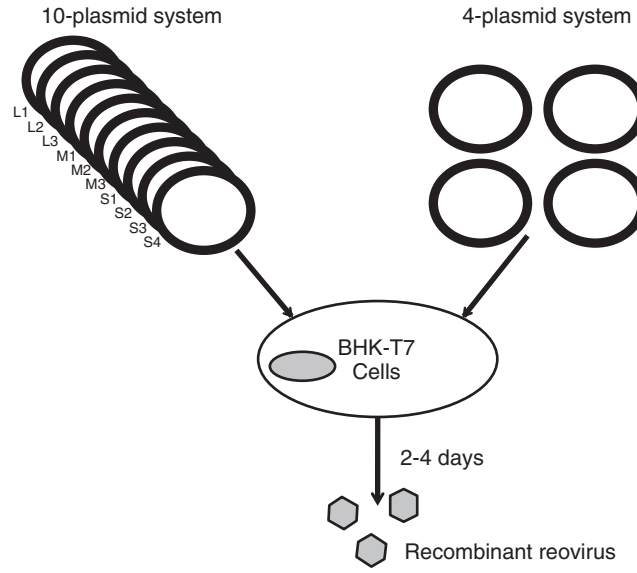

Fig. 2 Reverse genetics for recombinant reovirus rescue. Using the ten- or four-plasmid system, BHK-T7 cells are transfected with plasmids containing reovirus cDNA. The cells are incubated at 37 °C for 2–4 days and then lysed by multiple freeze/thaw cycles to harvest recombinant reovirus

Coinfection of cells with two distinct reovirus serotypes produces reassortant viruses, which are progeny viruses that contain different combinations of gene segments from the parental strains. Panels of reassortant viruses with known genomic content can be tested for the capacity to elicit a specific phenotype. Statistical analysis is employed to determine which gene or genes associate with a particular phenotypic effect. Reassortant reoviruses can be generated by plasmid-based reverse genetics system by blending the desired combination of plasmids. Single-gene reassortant viruses can be produced by individually replacing a gene segment in one genetic background with a single-gene segment from a different reovirus strain (Fig. 3). More genetically complex reassortant panels can be created from pools of viruses that contain multiple gene segments from each parental strain. Gene segments associated with a specific phenotype can be identified using the same analyses applied to traditional reassortant panels.

2 Materials

2.1 Cell Lines and Reagents

All cell culture reagents should be sterile.

1. Baby hamster kidney (BHK-21) cell line that constitutively expresses bacteriophage T7 RNA polymerase (BHK-T7) [20] (*see* **Note 1**).

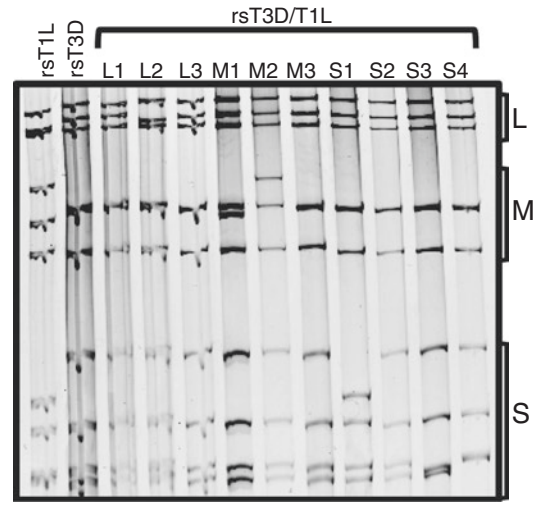

Fig. 3 Electrophoretic analysis of a reovirus single-gene reassortant panel. Purified virions were electrophoresed in a 10% SDS-polyacrylamide gel, followed by ethidium bromide staining (0.5 μg/mL) to visualize viral dsRNA gene segments. Shown are recombinant wild-type strains rsT1L and rsT3D, along with ten single-gene reassortants in which a single-gene segment from T3D was replaced with a gene segment from T1L. The size classes of the large, medium, and small gene segments are indicated as L, M, and S, respectively

2. Spinner-adapted mouse L929 cells.
3. Complete Dulbecco's modified Eagle's MEM (DMEM) (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen), 2 mM L-glutamine (Invitrogen), 100 U/mL of penicillin + 100 μg/mL of streptomycin mixture (Invitrogen), and 250 ng/mL of amphotericin B (Sigma). Store at 4 °C.
4. OPTI-MEM I reduced serum medium (Invitrogen). Store at 4 °C.
5. Complete Joklik's MEM (JMEM) (Sigma) supplemented with 5% fetal bovine serum, 2 mM glutamine, 100 U/mL of penicillin + 100 μg/mL of streptomycin mixture, and 250 ng/mL amphotericin B. Store at 4 °C.
6. Double concentration (2×) Med199 medium (Sigma), incomplete (*see* **Note 2**). Store at 4 °C.
7. Complete 2× Med199 medium supplemented with 5% fetal bovine serum, 4 mM L-glutamine, 200 U/mL penicillin + 200 μg/mL of streptomycin mixture, and 500 ng/mL of amphotericin B. Store at 4 °C.
8. Geneticin® (Invitrogen).
9. 2% Bacto-Agar solution (Fisher Scientific) (*see* **Note 3**).